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MUC1-C Oncoprotein Induces TCF7L2 Transcription Factor Activation and Promotes Cyclin D1 Expression in Human Breast Cancer Cells*[S]

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Background: MUC1-C oncoprotein has been linked to β-catenin-dependent gene activation by mechanisms that are unclear.
Results: MUC1-C binds directly to the TCF7L2 transcription factor and recruits β-catenin to the cyclin D1 promoter.
Conclusion: MUC1-C induces cyclin D1 expression in breast cancer cells by activating TCF7L2-mediated transcription.
Significance: Targeting MUC1-C function down-regulates cyclin D1 overexpression in breast cancer cells.

MUC1 is a heterodimeric glycoprotein that is overexpressed in breast cancers. The present studies demonstrate that the oncogenic MUC1 C-terminal subunit (MUC1-C) associates with the TCF7L2 transcription factor. The MUC1-C cytoplasmic domain (MUC1-CD) binds directly to the TCF7L2 C-terminal region. MUC1-C blocks the interaction between TCF7L2 and the C-terminal-binding protein (CtBP), a suppressor of TCF7L2-mediated transcription. TCF7L2 and MUC1-C form a complex on the cyclin D1 gene promoter and MUC1-C promotes TCF7L2-mediated transcription by the recruitment of β-catenin and p300. Silencing MUC1-C in human breast cancer cells down-regulated activation of the cyclin D1 promoter and decreased cyclin D1 expression. In addition, a MUC1-C inhibitor blocked the interaction with TCF7L2 and suppressed cyclin D1 levels. These findings indicate that the MUC1-C oncoprotein contributes to TCF7L2 activation and thereby promotes cyclin D1 expression in breast cancer cells.

The canonical Wnt pathway is of importance to developmental cell fate and tumorigenesis (1, 2). Activation of Wnt signaling is associated with inhibition of glycogen synthase kinase 3β (GSK3β)[3] and thereby the stabilization and accumulation of β-catenin, a component of the adherens junction of mammalian epithelial cells. Free β-catenin also forms complexes with members of the T-cell factor/leukocyte enhancing factor 1 (TCF/LEF1) family of high mobility group (HMG) transcription factors (3, 4). The mammalian TCF family members (TCF1, LEF1, TCF3, and TCF4/TCF7L2) bind to Wnt-responsive DNA elements (CCTTTG(A/T)(A/T)) and suppress gene transcription through interactions with the Groucho repressor (3, 4). Stabilization of β-catenin in response to Wnt activation promotes binding of β-catenin to TCFs, which, in turn, displaces Groucho (5). The interaction between β-catenin and the TCF N-terminal domain also recruits coactivators, such as CREB-binding protein/p300, BCL9, and Pygo, with the induction of gene transcription (3, 4). In this model, certain TCFs, for example TCF7L2, thus function as transcriptional repressors or activators of Wnt target genes in a manner dictated by the availability of nuclear β-catenin. TCF family members consist of four domains: an N-terminal β-catenin binding region, a central domain, a HMG DNA-binding domain, and a C-terminal tail (3). Alternative splicing at the 3′-end of TCF mRNAs generates protein isoforms with variations in the C-terminal region. In this regard, one of the TCF7L2 isoforms contains an E-tail that is necessary for full activity (6–8). The E-tail includes a 30-amino acid motif that is highly conserved from flies to humans, suggesting that it fulfills an important role (9). Indeed, within the E-tail is a conserved cluster of cysteines flanked by basic residues, known as the cysteine clamp (C-clamp), which functions as an auxiliary DNA-binding domain and is involved in recruitment of the transcriptional coactivator p300 (7, 10). The TCF E-tail also contains binding motifs for C-terminal-binding proteins (CtBPs) that function as transcriptional repressors (11–13).

The mucin 1 (MUC1) transmembrane glycoprotein is overexpressed aberrantly in ~90% of human breast cancers (14). MUC1 normally is found on the apical borders of mammary epithelial cells; however, with transformation and loss of polarity, MUC1 expression is up-regulated over the entire cell membrane (14, 15). Following translation, the MUC1 protein undergoes autocleavage into two subunits that in turn form a heterodimeric complex at the cell membrane (14). The MUC1 N-terminal subunit (MUC1-N) contains the extensively glycosylated tandem repeats that are characteristic of the mucin family and contribute to a physical barrier at the cell surface. The MUC1 C-terminal subunit (MUC1-C) is the transmembrane component of the heterodimer that also accumulates in the cytosol of transformed cells and is targeted to the nucleus (14). The MUC1-C subunit includes a 72-amino acid cytoplasmic domain, which contains a serine-rich motif with homology to

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[3] The abbreviations used are: GSK3β, glycogen synthase kinase 3; TCF/LEF1, T-cell factor/leukocyte enhancing factor 1; MUC1-CD, MUC1-C cytoplasmic domain; CtBP, C-terminal-binding protein; HMG, high mobility group; CREB, cAMP-responsive element-binding protein; qPCR, quantitative PCR.

sequences in E-cadherin and the adenomatous polyposis coli protein that function as β-catenin binding sites (16, 17). In this context, the MUC1-C cytoplasmic domain also binds to β-catenin Armadillo repeats and this interaction inhibits β-catenin degradation (16, 18). Moreover, the MUC1-C cytoplasmic domain functions as a substrate for GSK3β (19) and blocks GSK3β-mediated phosphorylation of β-catenin (18). Importantly, overexpression of the MUC1-C cytoplasmic domain induces anchorage-independent growth and tumorigenicity, which is attenuated by mutating the serine-rich motif binding site and thereby disruption of the MUC1-C/β-catenin interaction (18). The relevance of this MUC1-mediated pathway is further supported by the demonstration that the onset of *Wnt-1*-induced mammary tumors is decreased significantly in a Muc1-null background (20).

The present studies demonstrate that the MUC1-C cytoplasmic domain binds directly to the TCF7L2 E-tail and blocks binding of the CtBP repressor. The results show that MUC1-C is detectable with TCF7L2 on the promoter of cyclin D1 gene in human breast cancer cells and promotes cyclin D1 expression. We also show that treatment with a MUC1-C inhibitor suppresses cyclin D1 levels in human breast cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human ZR-75-1 and BT-549 breast cancer cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Human MCF-7 breast cancer cells were grown in DMEM with fetal bovine serum, antibiotics, and L-glutamine. Cells were treated with the MUC1-C inhibitor GO-201 or the control peptide CP-1 as described (21). Cells were also infected with lentiviruses expressing MUC1 or MUC1(AQA) (22).

Immunoprecipitation and Immunoblotting—Lysates from subconfluent cells were prepared as described (23). Soluble proteins (500 μg) were precipitated with anti-TCF7L2 (sc-13027, 1:50; Santa Cruz Biotechnology). Immunoprecipitates and cell lysates (40 μg) were subjected to immunoblotting with anti-MUC1-C (Ab5, 1:1000; LabVision), anti-TCF7L2 (1:1000), anti-cyclin D1 (Ab3, 1:1000; LabVision), anti-CtBP (1:1000; Santa Cruz Biotechnology), anti-β-catenin (610154, 1:1000; BD Biosciences), and anti-β-actin (1:10,000; Sigma). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

In Vitro Binding Assays—GST, GST-TCF7L2, GST-MUC1-CD, GST-MUC1-CD(1–45), GST-MUC1-CD(46–72) (23), GST-MUC1-CD(AQA), GST-MUC1-CD(CQA), and MUC1-CD(AQC) (24) were prepared as described. GST-CtBP was generated and prepared as described (25). Purified GST-TCF7L2, GST-MUC1-CD, GST-β-catenin, and GST-CtBP proteins were cleaved with thrombin to remove the GST moiety. GST proteins were incubated with MUC1-CD (17 μg) or TCF7L2 (21 μg) for 2 h at room temperature. In certain experiments, (i) GST and GST-MUC1-CD were incubated with β-catenin (2 μg) in the absence and presence of TCF7L2 (8 μg), and (ii) GST and GST-CtBP were incubated with TCF7L2 (8 μg) in the absence and presence of MUC1-CD (17 μg). Adsorbates to glutathione-conjugated beads were analyzed by immunoblotting.

Promoter-luciferase Reporter Assays—Cells (5×10^5) growing in six-well plates were transfected with 1 μg of pcycD1(−161)-Luc (17) and 1 ng of SV-40-*Renilla*-Luc in the presence of Lipofectamine. At 48 h after transfection, the cells were lysed in passive lysis buffer. Lysates were analyzed for firefly and *Renilla* luciferase activities with the Dual-Luciferase assay kit (Promega).

RT-PCR—Total RNA was isolated from cells using the RNeasy mini kit (Qiagen). RNAs were analyzed using the One-Step RT-PCR kit with Platinum Taq (Invitrogen). Primers used for RT-PCR are listed in supplemental Table 1.

ChIP Assays—Soluble chromatin was prepared from 2–3 × 10^6 cells as described (24) and precipitated with anti-TCF7L2 (2 μg), anti-HDAC1 (ab-7028, 2 μg; Abcam), anti-p300 (sc-584, 2 μg; Santa Cruz Biotechnology), anti-histone H3K9Ac (ab-4441, 2 μg; Abcam), or a control nonimmune IgG (2 μg). For re-ChIP assays, complexes from the initial ChIP were eluted and reimmunoprecipitated with anti-β-catenin (2 μg), anti-MUC1-C (2 μg), or anti-p300 (2 μg) as described (24). For PCR, 2 μl from a 50-μl DNA sample was used with the indicated primers (supplemental Table 2) and 25–35 cycles of amplification.

RESULTS

MUC1-C Cytoplasmic Domain Associates with TCF7L2—Previous work showed that the MUC1-C subunit binds directly to the β-catenin Armadillo repeats (18). β-catenin forms a transcriptional complex with TCF7L2; however, it is not known whether MUC1-C interacts with TCF7L2. Using lysates from ZR-75-1 breast cancer cells, immunoblot analysis of anti-TCF7L2 precipitates with anti-MUC1-C demonstrated that MUC1-C associates with TCF7L2 (Fig. 1*A*, *left*). Similar results were obtained when analyzing MCF-7 breast cancer cell lysates (Fig. 1*A*, *right*). MUC1-C includes a 72-amino acid cytoplasmic domain (MUC1-CD) that contains a β-catenin binding motif (Fig. 1*B*). Incubation of ZR-75-1 cell lysates with a GST-MUC1-CD fusion protein further demonstrated an association with TCF7L2 (Fig. 1*C*, *left*). These results were confirmed with MCF-7 cell lysates (Fig. 1*C*, *right*), indicating that the MUC1-C cytoplasmic domain is sufficient for conferring the TCF7L2 interaction. To determine whether the MUC1-C cytoplasmic domain interacts directly with TCF7L2, GST and GST-TCF7L2 were incubated with purified MUC1-CD. Binding of MUC1-CD was detectable with GST-TCF7L2 and not GST (Fig. 1*D*). Incubation of GST-MUC1-CD with purified TCF7L2 further supported a direct interaction (Fig. 1*E*). These results indicated that MUC1-CD binds directly to TCF7L2.

MUC1-C Cytoplasmic Domain CQC Motif Interacts with TCF7L2 E-tail—To define the MUC1-CD amino acids responsible for the interaction, we first incubated MUC1-CD(1–45) and MUC1-CD(46–72) with TCF7L2. MUC1-CD(1–45), but not MUC1-CD(46–72), bound to TCF7L2 (Fig. 2*A*). MUC1-CD contains a CQC motif at residues 1–3. Mutation of both Cys residues to Ala (AQA) blocked the interaction between MUC1-CD and TCF7L2 (Fig. 2*B*). In addition, individual mutations of MUC1-CD Cys-1 → Ala (AQC) or Cys-3 → Ala (CQA) decreased binding to TCF7L2, indicating that both

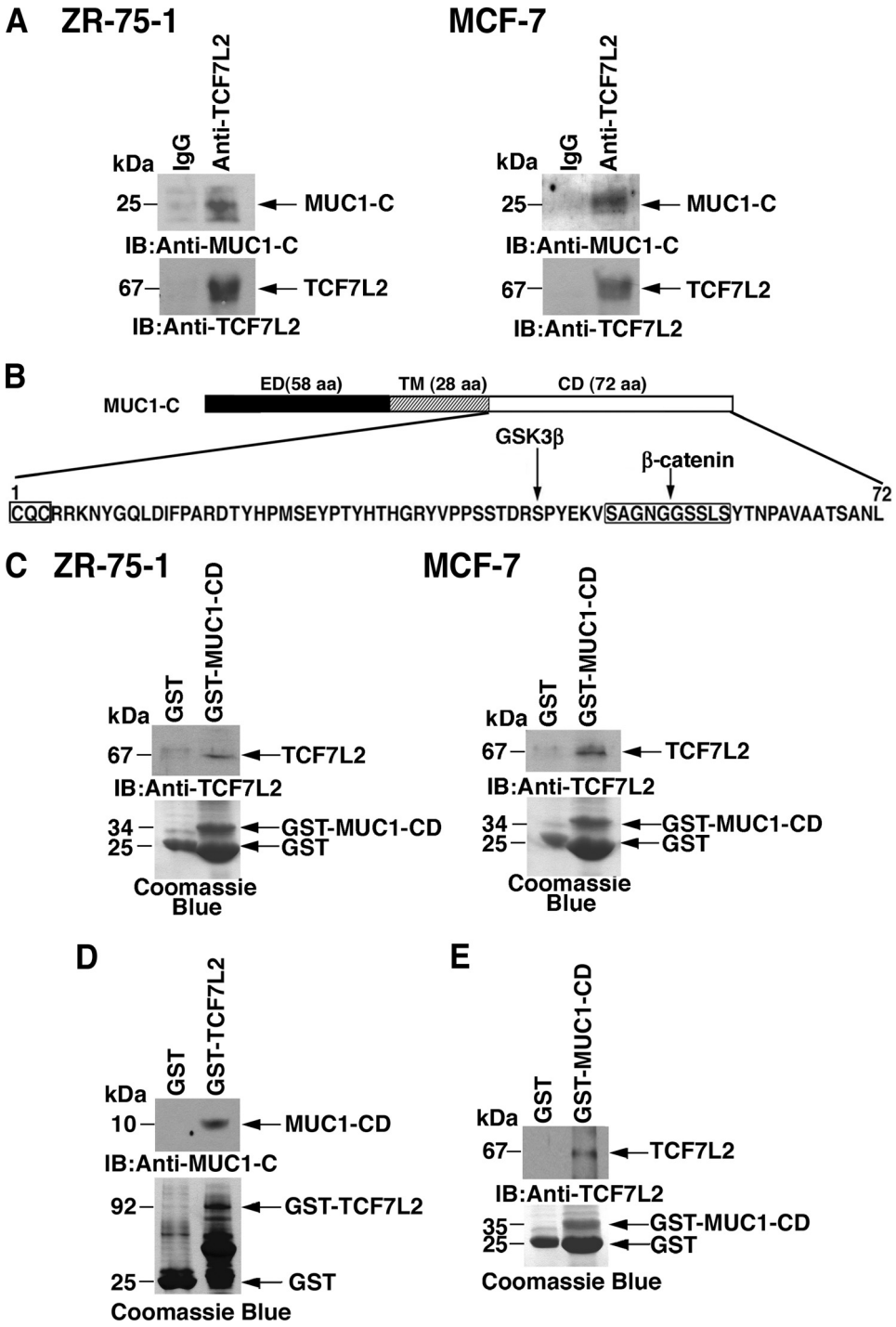

FIGURE 1. **MUC1-C cytoplasmic domain associates with TCF7L2.** *A*, lysates from ZR-75-1 (*left*) and MCF-7 (*right*) breast cancer cells were precipitated with anti-TCF7L2 or, as a control, non-immune IgG. The precipitates were immunoblotted with the indicated antibodies. *B*, schematic representation of MUC1-C (*ED*, extracellular domain; *TM*, transmembrane region) and the amino acid (*aa*) sequence of the cytoplasmic domain (*CD*). Highlighted are the CQC motif, the GSK3β phosphorylation site, and the β-catenin binding region. *C*, lysates from ZR-75-1 (*left*) and MCF-7 (*right*) cells were incubated with GST or GST-MUC1-CD. The adsorbates were immunoblotted with anti-TCF7L2. *Input* of the GST proteins was assessed by Coomassie Blue staining. *D* and *E*, GST or GST-TCF7L2 was incubated with purified MUC1-CD (*D*). GST or GST-MUC1-CD was incubated with recombinant TCF7L2 (*E*). Adsorbates were immunoblotted (*IB*) with the indicated antibodies. *Input* of the GST proteins was assessed by Coomassie Blue staining.

Cys residues contribute to the interaction (Fig. 2*B*). Human TCF7L2 consists of 596 amino acids, which includes an N-terminal β-catenin binding region, HMG/DNA binding domain, and an E-tail C-terminal region (Fig. 2*C*). To define the region(s) responsible for the interaction with MUC1-C, GST-TCF7L2 fragments were generated to include amino acids 1–294, 295–476, and 477–596 (Fig. 2*C*). Incubation of the GST-TCF7L2 fragments with MUC1-CD demonstrated direct binding to TCF7L2(295–476) and TCF7L2(477–596), but not the TCF7L2(1–294) fragment that contains the β-catenin-binding site (Fig. 2*D*). These results and the previous demonstration that β-catenin binds to MUC1-CD at a serine-rich

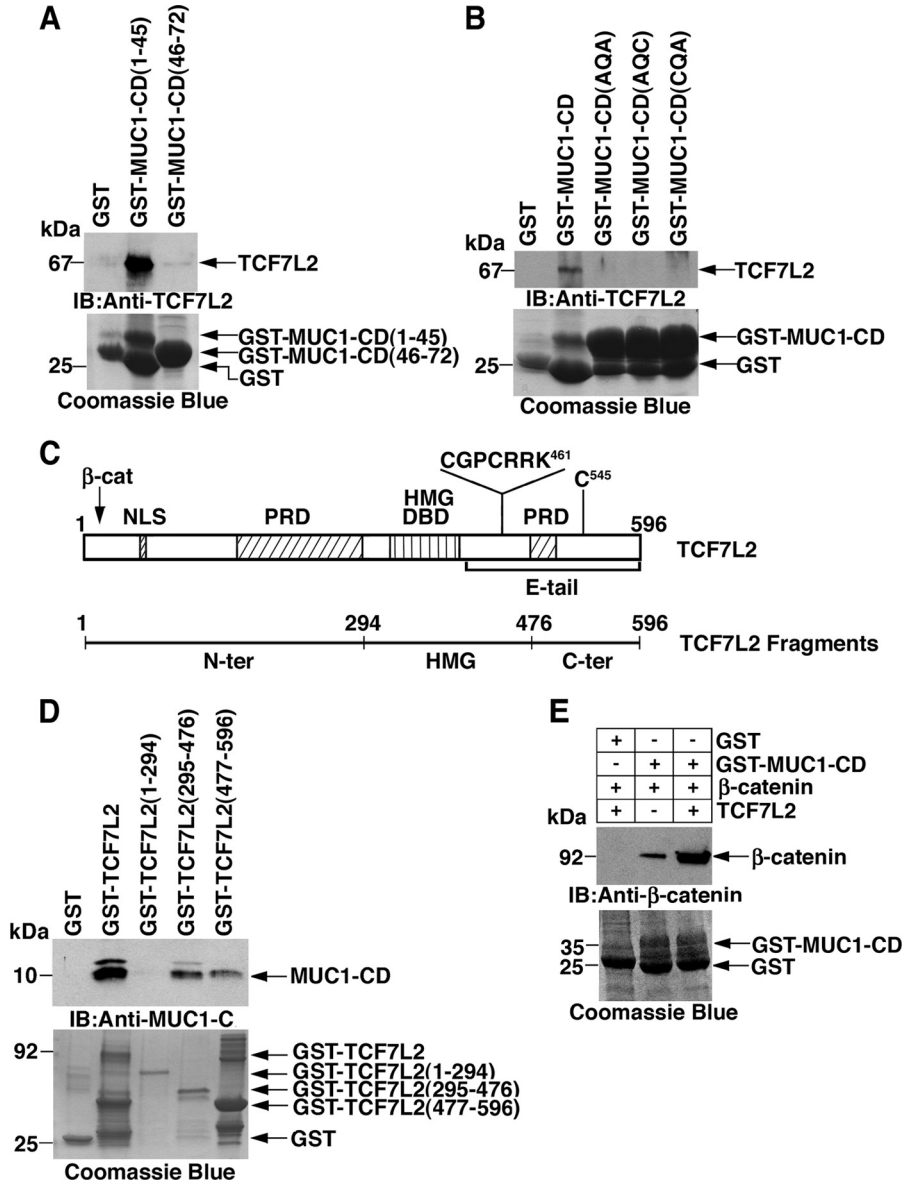

FIGURE 2. **MUC1-CD CQC motif confers binding to TCF7L2.** *A*, GST, GST-MUC1-CD(1–45), or GST-MUC1-CD(46–72) were incubated with recombinant TCF7L2. Adsorbates were immunoblotted (*IB*) with anti-TCF7L2. *Input* of the GST proteins was assessed by Coomassie Blue staining. *B*, GST or the indicated GST-MUC1-CD proteins were incubated with recombinant TCF7L2. Adsorbates were immunoblotted with anti-TCF7L2. *Input* of the GST proteins was assessed by Coomassie Blue staining. *C*, schematic representation of the TCF7L2 protein. Highlighted are the β-catenin (*β-cat*) binding region, the proline-rich domains (*PRD*), the HMG DNA-binding domain (*DBD*), and the E-tail that begins just downstream to the HMG/DBD and contains the Cys-rich C-clamp. *D*, GST or the indicated GST-TCF7L2 proteins were incubated with MUC1-CD. The adsorbates were immunoblotted with anti-MUC1-C. *Input* of the GST proteins was assessed by Coomassie Blue staining. *E*, GST or GST-MUC1-CD was incubated with β-catenin in the absence and presence of TCF7L2. Adsorbates were immunoblotted with anti-β-catenin. *Input* of the GST proteins was assessed by Coomassie Blue staining. *N-ter*, N-terminal; *C-ter*, C-terminal. *NLS*, nuclear localization signal.

sequence downstream from the CQC motif invoked the possibility that MUC1-CD could bind to both TCF7L2 and β-catenin. As shown previously (16, 18), MUC1-CD was found to interact with β-catenin (Fig. 2*E*). Moreover, the addition of TCF7L2 was associated with a marked increase in MUC1-CD-β-catenin complexes (Fig. 2*E*), indicating that MUC1-CD, TCF7L2, and β-catenin form a ternary complex.

MUC1-C Cytoplasmic Domain CQC Motif Binds Directly to TCF7L2 C-clamp—To confirm that the MUC1-CD CQC motif is responsible for the interaction with TCF7L2(295–476), we showed that, in contrast to MUC1-CD, there was no detectable binding to MUC1-CD(AQA) (Fig. 3*A*). Moreover, in studies with the TCF7L2(477–596) fragment, we found that binding of MUC1-CD is blocked by alteration of the CQC motif to AQA (Fig. 3*B*), indicating that the MUC1-CD Cys residues are responsible for the interactions with TCF7L2(295–476) and TCF7L2(477–596). TCF7L2(295–476) contains a conserved CGPCRRK motif (amino acids 454 to 461) in the C-clamp (7), which could represent a potential binding site for the MUC1-CD CQC motif. In this capacity, mutation of CGPCRRK to AGPARRK abrogated the interaction between TCF7L2(295–476) and MUC1-CD (Fig. 3*C*, *left*), supporting a model in which the MUC1-CD CQCRRK motif interacts directly with CGPCRRK (Fig. 3*C*, *right*). With regard to TCF7L2(477–596), this fragment contains a single Cys residue at position 545. Mutation of Cys-545 to Ala (C545A), however,

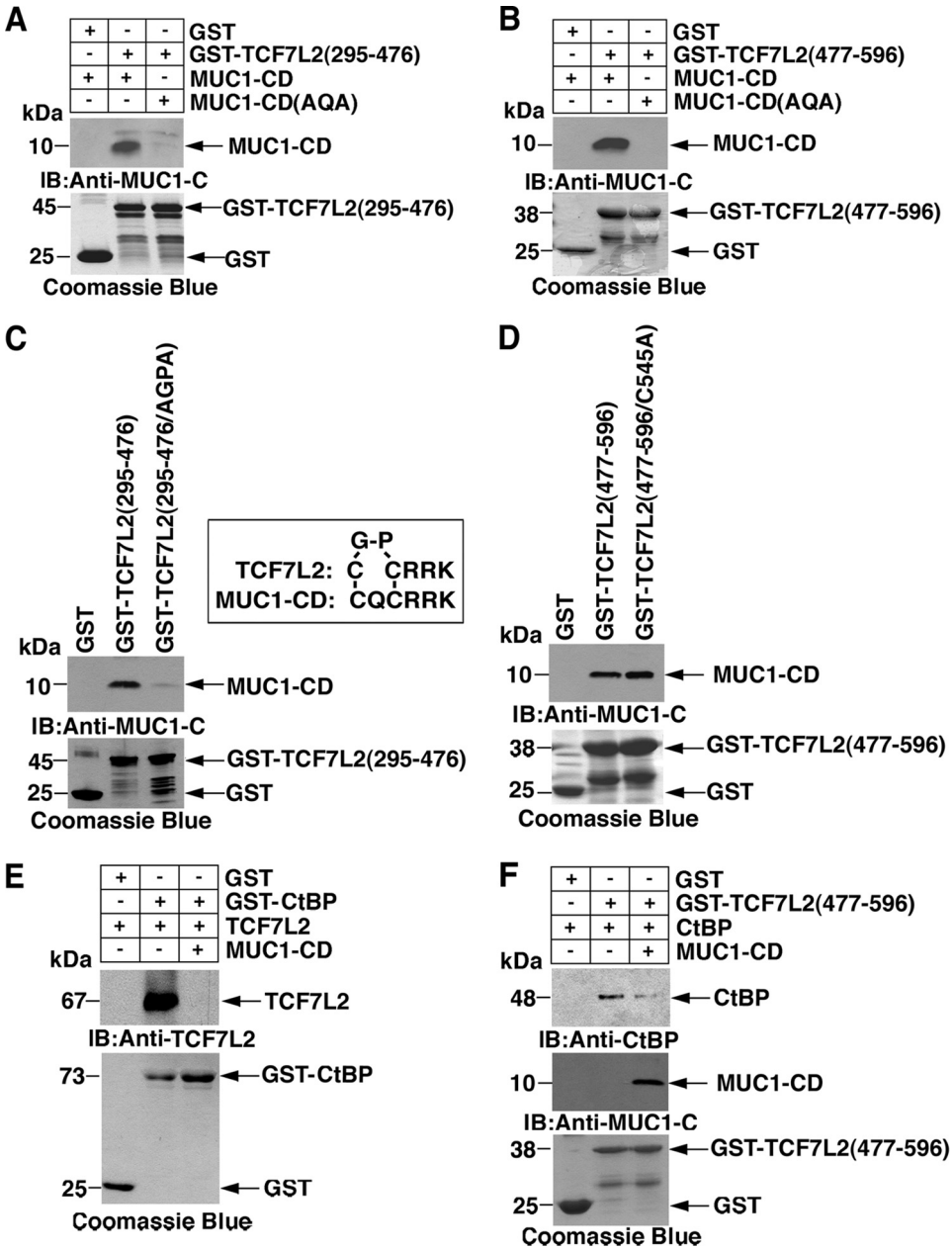

FIGURE 3. **MUC1-CD binds directly to the TCF7L2 CGPCRRK motif in the E-tail.** *A* and *B*, GST-TCF7L2(295–476) (*A*) or GST-TCF7L2(477–596) (*B*) was incubated with MUC1-CD or MUC1-CD(AQA). The adsorbates were immunoblotted with anti-MUC1-C. *Input* of the GST proteins was assessed by Coomassie Blue staining. *C*, GST or the indicated wild-type or mutant GST-TCF7L2(295–476) proteins were incubated with MUC1-CD (*left*). The adsorbates were immunoblotted (*IB*) with anti-MUC1-C. *Input* of the GST proteins was assessed by Coomassie Blue staining. Shown is a proposed model for the binding of TCF7L2 CGPCRRK and MUC1-CD CQCRRK (*right*). *D*, GST or the indicated wild-type or mutant GST-TCF7L2(477–596) proteins were incubated with MUC1-CD. The adsorbates were immunoblotted with anti-MUC1-C. *Input* of the GST proteins was assessed by Coomassie Blue staining. *E*, GST and GST-CtBP were incubated with TCF7L2 in the absence and presence of MUC1-CD. Adsorbates were immunoblotted with anti-TCF7L2. *F*, GST and GST-TCF7L2(477–596) were incubated with CtBP in the absence and presence of MUC1-CD. Adsorbates were immunoblotted with anti-CtBP and anti-MUC1-C. *Input* of the GST proteins was assessed by Coomassie Blue staining.

had no effect on the interaction between MUC1-CD and TCF7L2(477–596) (Fig. 3*D*). The TCF7L2 E-tail also contains two CtBP binding motifs that include the PLSL sequence and begin at positions 510 and 587 (12). To determine whether the interaction between MUC1-CD and TCF7L2 affects CtBP binding, we first incubated GST-CtBP with TCF7L2 in the absence and presence of MUC1-CD. As shown previously (12), TCF7L2 formed a complex with CtBP (Fig. 3*E*). The results further demonstrate that MUC1-CD blocks the TCF7L2-CtBP interaction (Fig. 3*E*). MUC1-CD also blocked the interaction between GST-TCF7L2(477–596) and CtBP (Fig. 3*F*). These findings indicate that the MUC1-CD CQC motif binds directly to the TCF7L2 C-terminal region and blocks the association with CtBP.

Association of TCF7L2 and MUC1-C on Cyclin D1 Promoter—To determine whether MUC1-C occupies a Wnt target gene with TCF7L2, we studied the promoter of the cyclin D1 gene that contains multiple TCF binding sites (Fig. 4*A*). As expected, ChIP studies demonstrated TCF7L2 occupancy of the cyclin D1 promoter TCF binding region in ZR-75-1 cells (Fig. 4*B*, *left*).

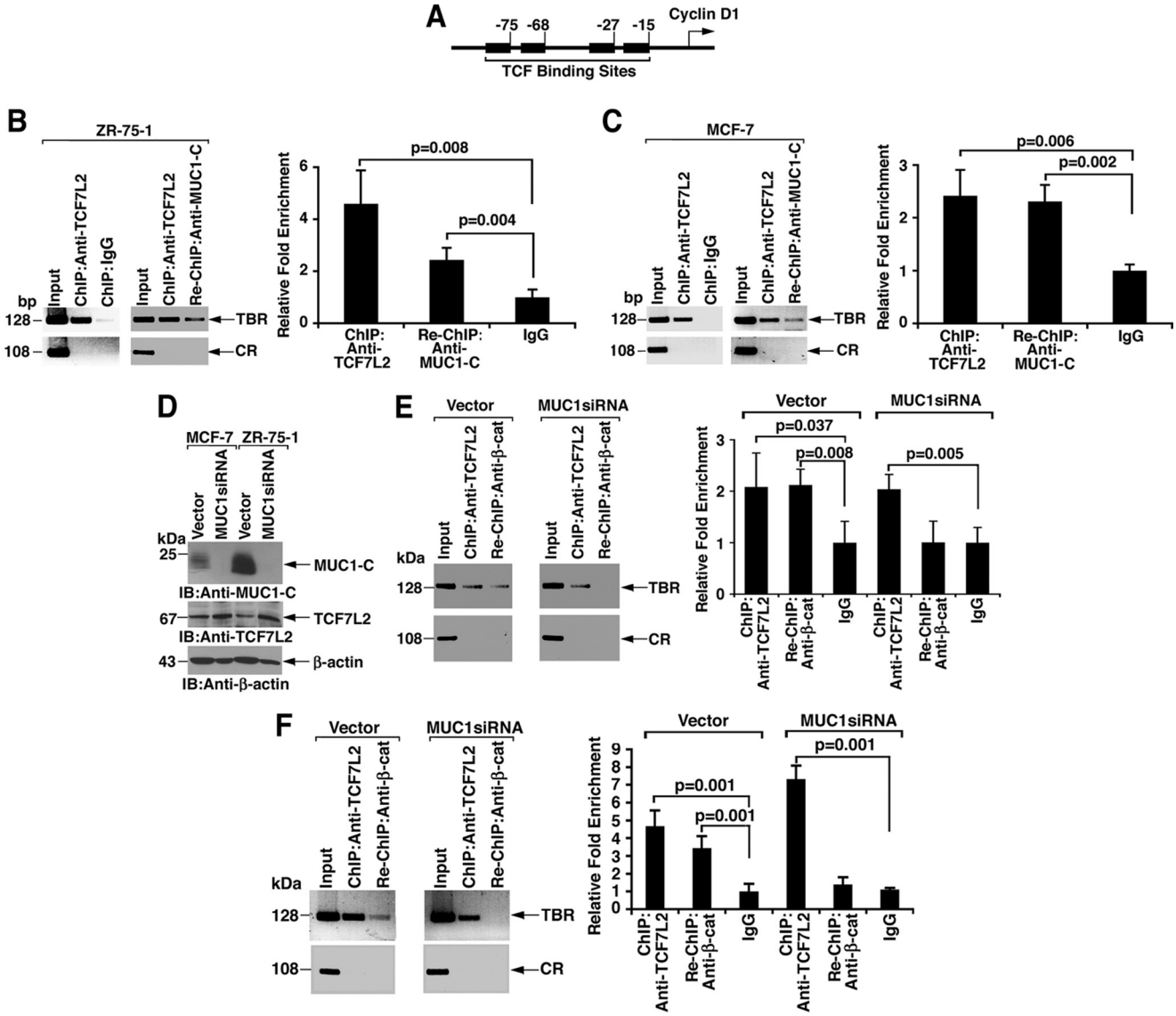

FIGURE 4. **MUC1-C occupies the cyclin D1 promoter in a complex with TCF7L2.** *A*, schematic representation of the cyclin D1 promoter with positioning of the TCF binding sites. *B* and *C*, soluble chromatin from ZR-75-1 (*B*) and MCF-7 (*C*) cells was precipitated with anti-TCF7L2 and, as a control, IgG (*left*). In the re-ChIP experiments, anti-TCF7L2 precipitates were released, reimmunoprecipitated with anti-MUC1-C, and then analyzed for cyclin D1 promoter sequences. The final DNA samples were amplified by PCR (*left*) and qPCR (*right*) with pairs of primers for the TCF binding region (*TBR*; −582 to −454) or a control region (*CR*; −4756 to −4648). The results (mean ± S.D. of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (*right*). *D*, lysates from MCF-7 and ZR-75-1 cells stably transfected with an empty vector or one expressing a MUC1 siRNA were immunoblotted with the indicated antibodies. *E* and *F*, soluble chromatin from the indicated ZR-75-1 (*E*) and MCF-7 (*F*) cells was precipitated with anti-TCF7L2 and analyzed for cyclin D1 promoter TCF binding region or CR sequences. In the re-ChIP experiments, the anti-TCF7L2 precipitates were released, reimmunoprecipitated with anti-β-catenin, and then analyzed for cyclin D1 promoter sequences by PCR (*left*) and qPCR (*right*). The results (mean ± S.D. of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (*right*).

Re-ChIP analysis further demonstrated that TCF7L2 occupies this promoter with MUC1-C (Fig. 4*B*, *left*). ChIP qPCR analysis confirmed that TCF7L2 and MUC1-C form a complex on the cyclin D1 promoter (Fig. 4*B*, *right*). Similar results were obtained when analyzing TCF7L2 and MUC1-C occupancy of the cyclin D1 promoter in MCF-7 cells (Fig. 4*C*, *left* and *right*). To assess in part the functional significance of the TCF7L2/MUC1-C interaction, studies were performed using MCF-7 and ZR-75-1 cells with stable silencing of MUC1-C expression (Fig. 4*D*). Down-regulation of MUC1-C was associated with a modest increase in total TCF7L2 levels (Fig. 4*D*). Moreover, silencing MUC1-C in ZR-75-1 cells had little if any effect on TFC7L2 occupancy of the cyclin D1 promoter as detected by ChIP PCR (Fig. 4*E*, *left*) and qPCR (Fig. 4*E*, *right*). Notably, however, silencing MUC1-C was associated with decreased β-catenin occupancy as determined by re-ChIP assays (Fig. 4*E*, *left* and *right*). Studies in MCF-7 cells confirmed that silencing MUC1-C has no effect on TCF7L2 occupancy but decreases the formation of TCF7L2-β-catenin complexes on the cyclin D1 promoter (Fig. 4*F*, *left* and *right*).

MUC1-C Regulates Histone H3 Acetylation on Cyclin D1 Promoter—TCF7L2 recruits corepressors, such as CtBP and HDAC1, in the absence of β-catenin (26, 27). In contrast, binding of β-catenin to TCF7L2 is associated with displacement of corepressors (5) and recruitment of the histone acetyltransferase and coactivator p300 (28). To assess the effects of

MUC1-C on coactivators and corepressors, additional ChIP studies were performed on the cyclin D1 promoter using the same primers as in the above studies. Silencing of MUC1-C in ZR-75-1 cells was associated with an increase in HDAC1 occupancy as determined by qPCR (Fig. 5*A*). Similar results were obtained in MCF-7 cells with MUC1-C silencing (Fig. 5*B*). MUC1-C occupancy of the cyclin D1 promoter in ZR-75-1/vector and MCF-7/vector cells was also associated with the increased formation of TCF7L2 complexes with p300 compared with that obtained in cells with MUC1-C silencing. (Fig. 5, *C* and *D*). In concert with these results, the presence of MUC1-C conferred an increase in histone H3-K9 acetylation on the cyclin D1 promoter in both ZR-75-1/vector (Fig. 5*E*) and MCF-7/vector (Fig. 5*F*) cells. These findings support a model in which MUC1-C occupancy on the cyclin D1 promoter is associated with (i) the displacement of HDAC1, (ii) the recruitment of p300, and (iii) an increase in histone H3 acetylation.

MUC1-C Activates Cyclin D1 Gene Transcription—To assess the effects of MUC1-C on TCF7L2-mediated transcription, ZR-75-1/vector and ZR-75-1/MUC1siRNA cells were transfected with pGL3, as a control, or a reporter containing the proximal cyclin D1 promoter (−166 bp) with four TCF binding sites upstream to luciferase (pcycD1(−166)-Luc). MUC1-C silencing was associated with substantial down-regulation of pcycD1(−166)-Luc activity (Fig. 6*A*, *left*). A similar response was observed in MCF-7 cells (Fig. 6*A*, *right*). In concert with the decrease in cyclin D1 promoter activity, silencing MUC1-C in both ZR-75-1 and MCF-7 cells resulted in decreased cyclin D1 mRNA levels as determined by RT-PCR (Fig. 6*B*, *left* and *right*). Moreover, MUC1-C silencing was associated with marked down-regulation of cyclin D1 protein levels (Fig. 6*C*, *left* and *right*). Human BT-549 breast cancer cells express MUC1-C, but at lower levels than that found in ZR-75-1 and MCF-7 cells. Consequently, to further assess the relationship between MUC1-C and cyclin D1 expression, we generated BT-549 cells stably expressing an empty vector (GFP) or MUC1-C (Fig. 6*D*, *left*). Overexpression of MUC1-C was associated with increases in cyclin D1 mRNA and protein levels (Fig. 6*D*) by a mechanism involving, at least in part, activation of the cyclin D1 promoter (Fig. 6*E*). Overexpression of full-length MUC1 in BT-549 cells was also associated with up-regulation of cyclin D1 abundance (Fig. 6*F*). In addition, expression of MUC1(AQA) resulted in down-regulation of cyclin D1 levels, indicating that binding of MUC1-C to TCF7L2 is of importance in activation of this pathway (Fig. 6*F*).

Inhibition of MUC1-C Blocks Binding to TCF7L2 and Decreases Cyclin D1 in Human Breast Cancer Cells—The peptide GO-201 (Fig. 7*A*) binds directly to the MUC1-C CQC motif and thereby blocks reactivity of this site (21). By contrast, the CP-1 control peptide, in which the Cys residues have been altered to Ala, is inactive in binding to the MUC1-C CQC motif (Fig. 7*A*). Accordingly, GO-201, but not CP-1, blocked the *in vitro* interaction between GST-TCF7L2 and MUC1-CD, consistent with involvement of the MUC1-CD CQC motif (Fig. 7*A*). In concert with the effects on binding of MUC1-C and TCF7L2, treatment of ZR-75-1 cells with GO-201, but not the inactive control CP-1, was associated with suppression of cyclin D1 expression (Fig. 7*B*). Similar results were obtained in MCF-7

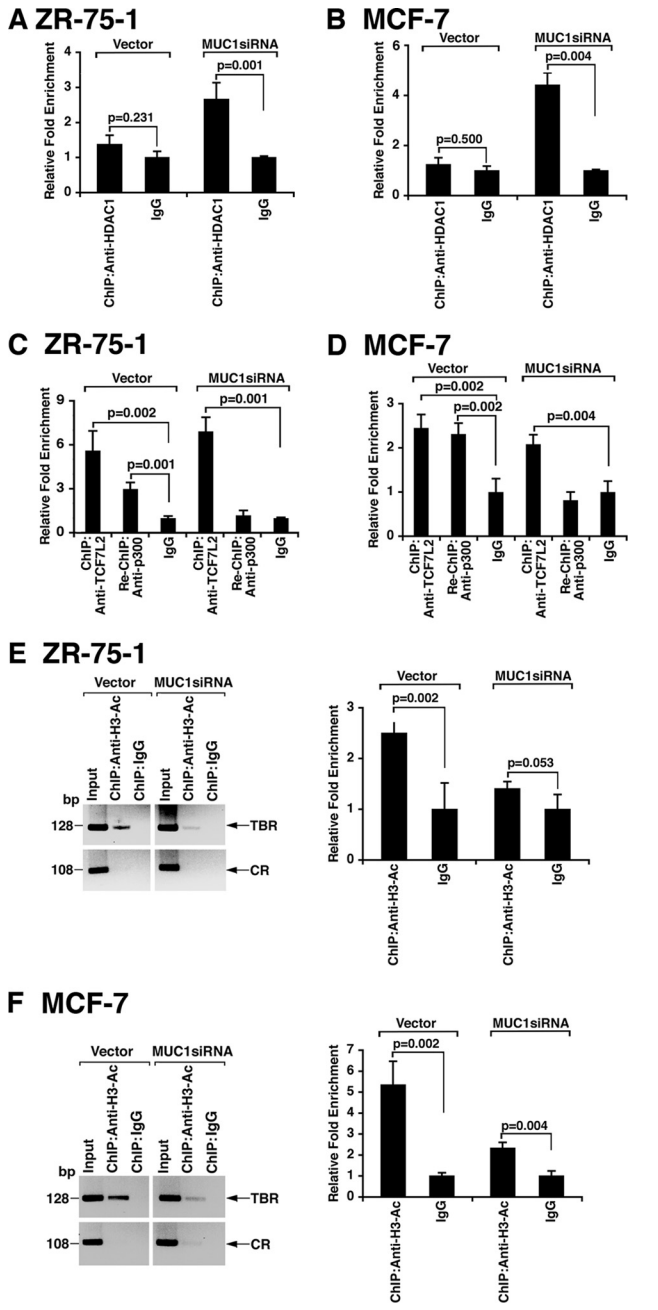

FIGURE 5. **MUC1-C promotes histone H3-K9 acetylation on the cyclin D1 promoter.** *A* and *B*, soluble chromatin from ZR-75-1 (*A*) and MCF-7 (*B*) cells was precipitated with anti-HDAC1 or a control IgG. The final DNA samples were amplified by qPCR with the same pairs of primers used for the cyclin D1 promoter in the studies shown in Fig. 4. The results (mean ± S.D. of three determinations) are expressed as the relative fold enrichment compared with that obtained with IgG control. *C* and *D*, soluble chromatin from ZR-75-1 (*C*) and MCF-7 (*D*) cells was precipitated with anti-TCF7L2. In the re-ChIP experiments, anti-TCF7L2 precipitates were released and reimunoprecipitated with anti-p300. The final DNA samples were amplified by qPCR with pairs of primers for the cyclin D1 promoter TCF binding region or a control region. The results (mean ± S.D. of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control. *E* and *F*, soluble chromatin from ZR-75-1 (*E*) and MCF-7 (*F*) cells was precipitated with anti-histone H3-K9-Ac or a control IgG. The final DNA samples were amplified by qPCR with pairs of primers for the cyclin D1 promoter TCF binding region or a control region (*left*). The results (mean ± S.D. of three determinations) are expressed as the relative fold enrichment compared with that obtained with IgG control (*right*).

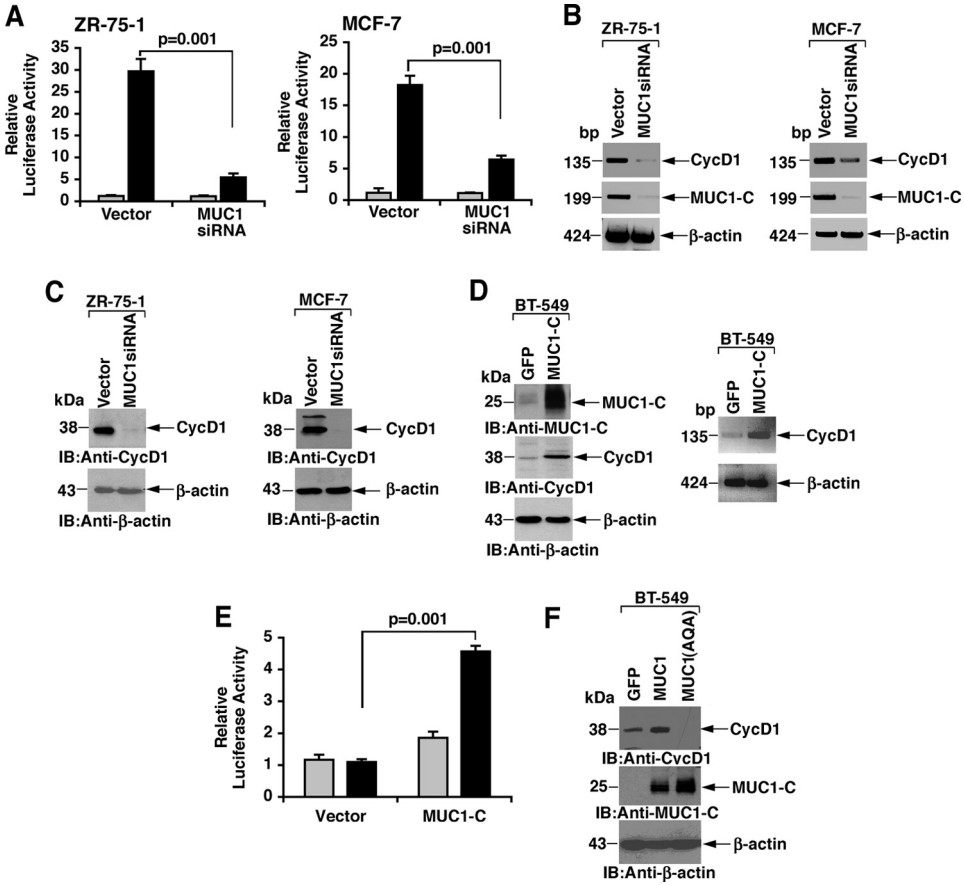

FIGURE 6. **MUC1-C promotes TCF7L2 activation of the cyclin D1 promoter.** *A*, the indicated ZR-75-1 (*left*) and MCF-7 (*right*) cells were transfected with pGL3 (*shaded bars*) or pGL3 expressing pcycD1(−166)-Luc (*solid bars*) for 48 h and then assayed for luciferase activity. The results are expressed as the relative luciferase activity (mean ± S.D. from three separate experiments) compared with that obtained with pGL3. *B*, RNA isolated from the indicated cells was analyzed by RT-PCR using primers designed to detect the indicated transcripts. *C*, lysates from the indicated ZR-75-1 (*left*) and MCF-7 (*right*) cells were immunoblotted with anti-cyclin D1 (*CycD1*) and anti-β-actin. *D*, BT-549 cells were infected with lentiviruses expressing GFP or MUC1-C. Lysates from the transduced cells were immunoblotted with the indicated antibodies (*left*). The indicated BT-549 cells were analyzed for cyclin D1 and β-actin mRNA levels by RT-PCR (*right*). *E*, the BT-549/GFP and BT-549/MUC1-C cells were transfected with pGL3 (*shaded bars*) or pGL3 expressing pcycD1(−166)-Luc (*solid bars*) for 48 h and then assayed for luciferase activity. The results are expressed as the relative luciferase activity (mean ± S.D. from three separate experiments) compared with that obtained with pGL3. *F*, BT-549 cells were infected with lentiviruses expressing GFP, MUC1, or MUC1(AQA). Lysates from the transduced cells were immunoblotted (*IB*) with the indicated antibodies.

cells treated with GO-201 (Fig. 7*C*). These results collectively provide support for model in which MUC1-C is of functional importance to the induction of cyclin D1 expression in human breast cancer cells (Fig. 7*D*).

DISCUSSION

MUC1-C Cytoplasmic Domain Associates with TCF7L2—Activation of the Wnt signaling pathway results in stabilization of β-catenin and thereby binding of β-catenin to TCF7L2 and other TCF family members. The MUC1-C subunit competes with E-cadherin for binding to β-catenin, interacts with the β-catenin Armadillo repeats and contributes to β-catenin stability (Fig. 7*D*) (14, 18). The present results demonstrate that the MUC1-C subunit associates with TCF7L2 in cells. *In vitro* binding studies further show that the MUC1-C cytoplasmic domain binds directly to TCF7L2 in the region C-terminal to the HMG/DNA binding domain known as the E-tail (7). The two Cys residues in the MUC1-C CQC motif were identified as being responsible for binding to the TCF7L2 E-tail. Few insights are available regarding the function of the TCF7L2 E-tail. The upstream 88-amino acid HMG domain consists of a 68-amino acid HMG box that recognizes the consensus Wnt-responsive DNA-binding sequence, and a nine-amino acid nuclear localization signal that participates in DNA binding (29). The TCF7L2 E-tail contains a 30-amino acid C-clamp that also binds to double-stranded DNA and promotes the interaction with Wnt-responsive elements (7). In addition, the C-clamp has been implicated in the recruitment of p300 to the transcription complex (10). Notably, the TCF7L2 C-clamp contains four conserved Cys residues, of which the two central cysteines (CGPC; amino acids 454–457) function as the binding site for the MUC1-C CQC motif. Accordingly, binding of the MUC1-C cytoplasmic domain to the TCF7L2 C-clamp could affect Wnt target gene recognition (7) or recruitment of p300 (10). The C-clamp is highly conserved in TCFs (7), indicating that MUC1-C may bind to other family members, such as TCF-1 and TCF-3, which also have E-tail isoforms. Our results indicate that the MUC1-C CQC motif also associates with the TCF7L2 E-tail at the C-terminal region (amino acids 477–596). This E-tail C-terminal region regulates TCF7L2-mediated Wnt target gene transcription by interacting with CtBP, a repressor of transcription (8, 30, 31). Notably, binding of the

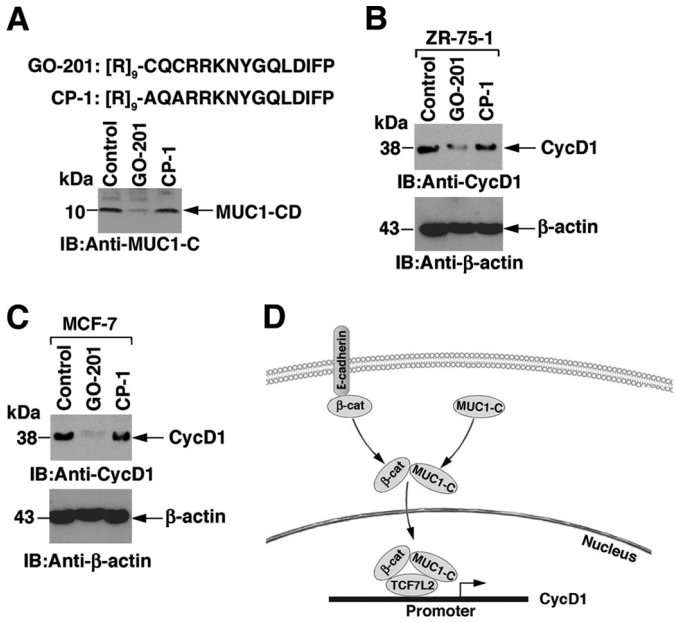

FIGURE 7. **Inhibition of MUC1-C decreases cyclin D1 expression.** *A*, amino acid sequences of the GO-201 and control CP-1 peptides. GST-TCF7L2 was incubated with MUC1-CD in the absence (*Control*) and presence of GO-201 or CP-1. Adsorbates were immunoblotted with anti-MUC1-C. *B* and *C*, ZR-75-1 (*B*) and MCF-7 (*C*) cells were left untreated (*Control*) and treated with 5 μM GO-201 or CP-1 each day for 3 days. Lysates were immunoblotted with the indicated antibodies. *D*, proposed model for the interaction of MUC1-C with TCF7L2 and β-catenin and thereby activation of the cyclin D1 promoter. MUC1-C competes with E-cadherin for binding to β-catenin (*β-cat*; 14) and forms a complex with β-catenin that localizes to the nucleus (18). In turn, MUC1-C promotes the formation of β-catenin-TCF7L2 complexes on the cyclin D1 (*CycD1*) promoter through binding to (i) the β-catenin Armadillo repeats and (ii) the TCF7L2 C-terminal region. *IB*, immunoblot.

MUC1-C cytoplasmic domain to the TCF7L2 C-terminal region blocked the interaction between TCF7L2 and CtBP, indicating that MUC1-C could relieve CtBP-mediated repression of TCF7L2 function.

MUC1-C Promotes TCF7L2-mediated Transcription of Cyclin D1 Gene—Cyclin D1 is an essential protein for activation of cyclin-dependent kinase partners and thereby cell cycle progression through the G_1/S phase transition (32). Computational analysis of gene expression patterns in diverse human tumor specimens had demonstrated that cyclin D1 overexpression is linked significantly to that of MUC1; however, it was not clear from those studies whether MUC1 functioned upstream or downsteam of cyclin D1 (33). The present results demonstrate that silencing MUC1-C in human ZR-75-1 and MCF-7 breast cancer cells is associated with down-regulation of cyclin D1 mRNA levels. Moreover, silencing MUC1-C was associated with suppression of cyclin D1 promoter activation, consistent with MUC1-C playing a functional role in enhancing cyclin D1 gene transcription. In concert with these observations, overexpression of MUC1-C in human BT-549 breast cancer cells resulted in activation of the cyclin D1 promoter and increased cyclin D1 expression. As expected, ChIP studies of the breast cancer cells demonstrated that TCF7L2 occupies the TCF binding sites of the cyclin D1 promoter. In addition, silencing MUC1-C had no apparent effect TCF7L2 occupancy. Consistent with the association of TCF7L2 and MUC1-C, re-ChIP studies further demonstrated that TCF7L2 occupies the TCF binding sites with MUC1-C. Previous work had shown that overexpression of MUC1-C is associated with increased localization of β-catenin to the nucleus (18). Notably, in the present work, silencing MUC1-C decreased β-catenin occupancy of the TCF binding sites, a result in concert with the suppression of cyclin D1 gene transcription. These findings could be explained by the effects of MUC1-C on the stabilization of β-catenin (18). Alternatively, the demonstration that MUC1-C occupies the cyclin D1 promoter with TCF7L2 raises the possibility that MUC1-C contributes to the formation of TCF7L2-β-catenin complexes on the TCF binding sites. In this regard, the MUC1-C CQC motif that binds to TCF7L2 is distinct from the MUC1-C serine-rich motif that is responsible for the interaction with β-catenin (Fig. 1*B*), thus providing a potential model in which MUC1-C could form a ternary complex with TCF7L2 and β-catenin, and thereby promote recruitment of coactivators, such as p300, and activation of cyclin D1 expression (Fig. 7*D*).

MUC1-C Cytoplasmic Domain Regulates Cyclin D1 Expression in Human Breast Cancer Cells—The Wnt pathway and cyclin D1 expression have been shown to be of importance to mammary tumorigenesis (34). For example, cyclin D1-deficient mice are resistant to the development of mammary cancers induced by the *erbB2* and *ras* oncogenes (35). By contrast, cyclin D1 is dispensable for c-*myc* or *Wnt-1*-driven mammary tumorigenesis, indicating that cyclin D1 may be of importance in only certain types of breast cancer (35). Other studies in mice expressing a mutant cyclin D1 deficient in activating CDK4/6 demonstrated resistance to ErbB2-initiated breast cancers (36). In addition, studies in a cyclin D1 kinase-deficient mouse have shown that cyclin D1 activity is necessary for self-renewal of mammary stem and progenitor cells that are the targets for ErbB2-mediated tumorigenesis (37). Notably, the cyclin D1 gene is amplified in ~20% of human breast cancers (38), and the cyclin D1 protein is expressed at increased levels in ~50% of human breast tumors (39–41). The human MUC1-C cytoplasmic domain is highly conserved in mice and other mammalian species (42). To determine whether inhibiting the MUC1-C cytoplasmic domain affects cyclin D1 expression, we used a cell-penetrating peptide, designated GO-201, which contains the CQCRRK sequence and blocks availability of the CQC motif for protein interactions (23, 24). In this way, GO-201 inhibited binding of the MUC1-C cytoplasmic domain to TCF7L2 and suppressed cyclin D1 expression in ZR-75-1 and MCF-7 breast cancer cells. Previous studies have shown that GO-201 treatment of nude mice bearing ZR-75-1 breast tumor xenografts is associated with inhibition of growth and regressions (21). Therefore, MUC1-C inhibitors, which have entered phase I clinical evaluation, could conceivably be effective against breast tumors that are dependent on Wnt signaling and cyclin D1 for their growth and survival. Finally, the present studies do not exclude the possibility that MUC1-C, which is a substrate for GSK3β (19), also contributes to the regulation of cyclin D1 expression by GSK3β-mediated mechanisms (43). In addition, recent studies have shown that cyclin D1 mediates the repair of damaged DNA (44), raising the possibility that inhibition of MUC1-C, and thereby suppression of cyclin D1, could

have an effect on DNA repair in the response to genotoxic anticancer agents.

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